MOLECULAR CYTOLOGY AND CYTOMETRY

HEAD
STANISLAV KOZUBEK

SCIENTISTS
EVA BÁRTOVÁ, EMILIE LUKÁŠOVÁ

RESEARCH FELLOWS
ALENA BAČÍKOVÁ, MARTIN FALK, PAVLA GAJDUŠKOVÁ, JANA KREJČÍ, LUDĚK STRAŠÁK

TECHNICIANS
JANA KŮROVÁ

GRADUATE STUDENTS
GABRIELA GALIOVÁ, ANDREA HARNIČAROVÁ

DIPLOMA STUDENTS
SOŇA LEGARTOVÁ

BACHELOR
RADKA UHLÍŘOVÁ

Nuclear topography and transcriptionalactivity of the c-myc gene during differentiation

Laboratory is focused on the studies on chromatin architecture in the view of gene expression that undergoes remarkable changes during various differentiation pathways. We tried to make correlations between observed structural phenomena and changes in epigenetic profiles such as histone acetylation and methylation. The highly conserved core histones H2A, H2B, H3 and H4, and their epigenetic modifications are thought to influence the coding and genetic potential of DNA. Epigenetics refers to heritable changes in the phenotype that occur irrespective of alterations in the DNA sequences. Therefore, the role of histone modification in gene silencing and/or X chromosome inactivation can be considered as an epigenetic process. Both euchromatin and heterochromatin are characterized by specific epigenetic patters and experiments related to structural and

functional characteristics of these genomic regions are performed in our laboratory.

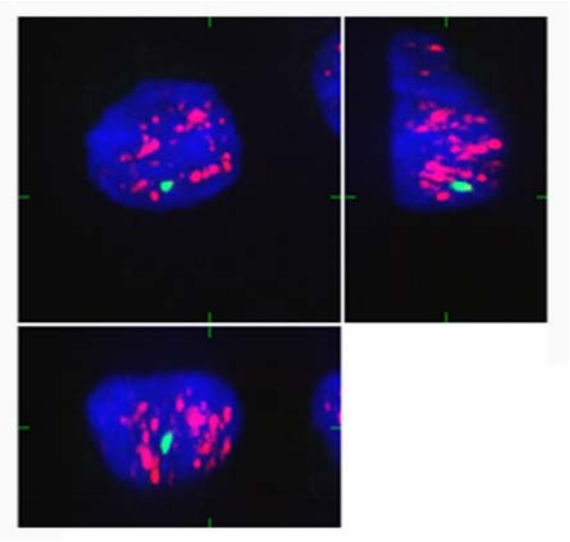

Fig. 1. 3D-projection of interphase nucleus of HT29 cell. Red regions are SC-35 domains involving splicing factors and green signals represent 3D-projection of c-myc transcription site.

Majority of our analyses were focused on nuclear location of the c-myc gene, and c-myc transcription sites in human adenocarcinoma HT29 cells. We have observed that the c-myc genes and its transcription sites were located non-randomly within the interphase nucleus, and c-myc RNA signals associated with the periphery of the centrally located nucleoli. Up-regulation of the APC gene reduced both the level of the c-MYC protein and the number of c-myc transcription sites. This correlated well with the decreased number of cells in the late G1- and S-phase of the cell cycle, during which the c-myc gene is mainly transcribed. A single transcription site of the c-myc gene co-localized from ~80% with the RNAP II region "transcription factories". However, in ~20% of cells, the c-myc transcriptional complex (green signal in enclosed figure) was released from the site of synthesis and did not co-localized with transcription factories. Partial association of c-myc transcripts with SC-35 domains "nuclear speckles" (red regions in enclosed figure) was observed. In majority of cells (~65% of nuclei), the c-myc RNA signals were located in close proximity to SC-35 regions, but promyelocytic leukaemia (PML) bodies adjoined to

transcription sites in only ~20% of nuclei. We suggest that c-myc gene transcription and c-myc pre-mRNA processing take place in close proximity to nucleoli, with the participation of factors contained in those SC-35 domains located in the most internal parts of the cell nucleus.

C-myc (8q24.21) nuclear arrangement was also studied in human embryonic stem cells (hES) that are unique in their pluripotency and capacity for self-renewal. Additionally, nuclear radial arrangement of Oct3/4 gene (6p21.33), responsible for hESC pluripotency, was analyzed. Unlike differentiated hES cells, pluripotent hES cell populations were characterized by a high level of decondensation for the territories of both chromosomes 6 (HSA6) and 8 (HSA8). The Oct3/4 genes were located on greatly extended chromatin loops in pluripotent hES cell nuclei, outside their respective chromosome territories (Fig. 2). However, this phenomenon was not observed for the Oct3/4 gene in differentiated hES cells and for the C-myc gene in the cell types studied. The high level of chromatin de-condensation in hES cells also influenced the nuclear distribution of all the variants of HP1 protein, particularly HP1β, which did not form distinct foci, as usually observed in most other cell types. Our experiments showed that unlike C-myc, the Oct3/4 gene and HP1 proteins undergo a high level of de-condensation in hES cells. Therefore, these structures seem to be primarily responsible for hES cell pluripotency due to their accessibility to regulatory molecules. Differentiated hES cells were characterized by a significantly different nuclear arrangement of the structures studied.

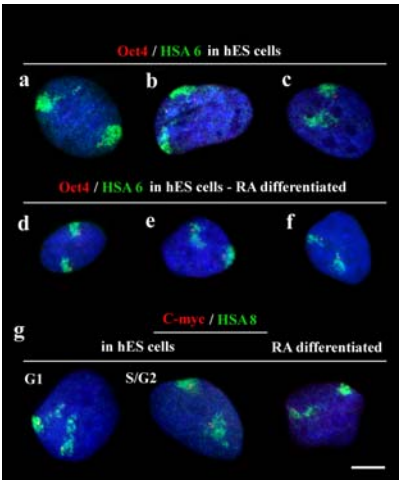

Fig. 2. Nuclear positionong of Oct3/4 (red) and c-myc (red) genes within interphase nuclei (blue) of pluripotent and RA-differentiated hESCs. Arrangement of selected genes was also analyzed in relationship to relevant chromosome territories (diffuse green) (Bártová et al., Differentiation, 2008). Bar shows 6 microm.

Studies on multiple myeloma

Part of our working group is engaged in the studies on multiple myeloma genetics and epigenetics. Chromosomal rearrangements can be frequently observed in tumour cells and multiple myeloma is not exception. Karyotypic abnormalities of MM are accompanied by changes in the epigenome, which preferentially involves aberrant DNA/histone methylation and histone acetylation, as well. We focused on analysis of epigenetic patterns in CD138+ and CD138- cells isolated from the bone marrow of patients with diagnosed MM. Additionally, we have studied the epigenetic changes in myeloma cells treated by clinically used cytostatics. In our experiments, chromatin immunoprecipitation (ChIP) in combination with polymerase chain reaction (PCR) enable us to analyze epigenetic profiles such as H3K9 acetylation and H3K9 di-methylation at promoters and coding regions of the genes which are important in pathogenesis of MM. In selected patients with diagnosed MM we observed different H3K9 acetylation and H3K9 dimethylation at c-myc and CCND1 sequences. Epigenetic changes in the c-myc and CCND1 genes were also studied in MM cells treated by melphalan, used in MM theraphy. ChIP-on-chip microarray analysis of H3K9 acetylation at promoter regions of hundreds of genes showed an increased H3K9 acetylation at promoters of many genes after melphalan treatment. This type of epigenetic modification is associated with an increased gene expression. Our data documented results of basic research, but on the other hand, we showed original ChIP-PCR and ChIP-on-chip analyses in clinical samples. Furthermore, we provided a useful tool how assess therapeutic potential of cytostatics, which significantly influence histone code.

Lamin A/C deficiency and chromatin structure

Diploma work of Gabriela Galiová is aimed at the study of lamin A/C deficiency and its effect on nuclear architecture. In article, accepted for publication in EJCB, we have shown that lamin A/C deficiency caused condensation of chromosome territories, which was compensated or even more pronounced by HDAC inhibition. Both lamin A/C deficiency and HDACi induced nuclear reorganization of centromeric heterochromatin, which was accompanied by the appearance of a chain-like morphology of HP1βfoci. Our observations lead to the suggestion that lamin A/C function could be connected to the reorganization of both hetero- and euchromatin as well as of some chromatin associated domains such as the HP1βfoci. Additionally, TSA can compensate the lamin A/C dependent chromatin changes, which support the statement that interaction between lamins and

specifically modified histones dictates nuclear architecture.

Chromatin structure influences the sensitivity of DNA to γ-radiation

The major advancement of this study is the direct evidence that ionizing radiation induces most double-strand breaks in genetically active, gene-dense regions of the human genome. Therefore, the most active regions of the human genome (structurally characterized by an open chromatin conformation) are subjected to the highest risk of radiation damage. On the other hand, we demonstrate the protective role of condensed chromatin, containing a low density of genes with low expression (functionally usually equivalent to heterochromatin). Higher sensitivity of open chromatin to damage is accompanied by more efficient DSB repair compared with the condensed chromatin. However, contrary to physiological heterochromatin, hypercondensed chromatin experimentally induced by hyperosmotic medium does not shield DNA against the damage by γ-radiation. Hypercondensed chromatin is probably formed by simple contraction of relaxed chromatin provoked by a decrease in the negative charge of the DNA in high concentration of salts without participation of additional chromatin-binding proteins, and therefore its sensitivity to ionizing radiation remains the same as that in cells with normal chromatin organization. To evaluate the radiosensitivity in functionally and structurally different chromatin domains we used ImmunoFISH technique, enabling the concomitant detection of DSBs as phosphorylated H2AX foci together with specific chromosomal regions. The most direct proofs follows from the comparison of DSB induction and repair in two regions of the same length (11 Mbp), located on chromosome 11 (separated by 12 Mbp) but markedly differing in the amount of highly expressed genes and therefore also in their chromatin structure. While the region RIDGE (region of increased gene expression) contains extremely high density of highly expressed genes and thus open chromatin structure, the anti-RIDGE has very low density of genes, mainly with low expression, and its chromatin is of 40 % more condensed than in RIDGE. The number of DSBs induced in the anti-RIDGE was of 76% lower and the repair was two times less effective compared the RIDGE. The same conclusions also follow from studies of DSB induction and distribution inside of individual chromosomal territories (CTs). Higher sensitivity to radiation damage was observed for chromosomes containing high density of genes (HSA19 and HSA11) contrary to those with low gene densities (HSA4, HSA18 and HSA2). This difference was, however, not so great (about 50%), probably because neither group of chromosomes contains only condensed or decondensed chromatin, unlike the RIDGE and anti-RIDGE.

The organization and structure of chromosome territories is maintained by nuclear lamina and polymeric actin

The role of lamina and nuclear actin in organization of chromosome territories (CT) was studied in cells of mammary carcinoma MCF7 by the damage of nuclear networks of these proteins either by inhibition of lamin A/C transcription or by preclusion of actin polymerization. The control of the efficiency of lamin A/C down regulation was pursued by RT-PCR, Western-blotting and immunodetection of the protein in individual nuclei. The actin filaments were detected by faloidin conjugated with rhodamine. CTs of HSA1, HSA2 and HSA13 were visualized by FISH using the painting probes conjugated with Cy3. The selected chromosomes differ in their length and in gene density but all of them are in contact with the nuclear membrane. All images obtained by confocal microscopy were deconvolved with Andor IQ 1.7 software (Andor Technology), using measured point-spread functions from Tetraspeck fluorescent beads (Molecular Probes-Invitrogen) and the Jansson-van Cittert deconvolution algorithm. To identify and quantitatively analyze FISH-labeled areas, deconvolved 3D images were treated with a bandpass filter and subsequently segmented using a range of thresholds in Huygens Essential software (Scientific Volume Imaging BV).

The disruption of the lamina in cells with silenced lamin A/C resulted in a decrease of the volume and surface area of chromosome territories, especially for chromosomes with low gene density and thus with high content of heterochromatin. The volume of CT2 and CT13 decreased of about 40%, while that of CT1 only of 17 %. Inhibition of actin polymerization led to significant enlargement of volumes and surface areas of chromosome territories. This increase was in correlation with gene density of chromosomes. The largest increase (150%) was observed for CT1, followed by that for CT2 (100%) and CT13 (70%).

These results show that lamina and nuclear polymeric actin, both participate on the maintaining of chromosome territories structure by different ways and independently each other. However changes induced in the size of CTs by disruption of one or another protein network are dependent on gene density determining the level of open chromatin structure of a particular chromosome.

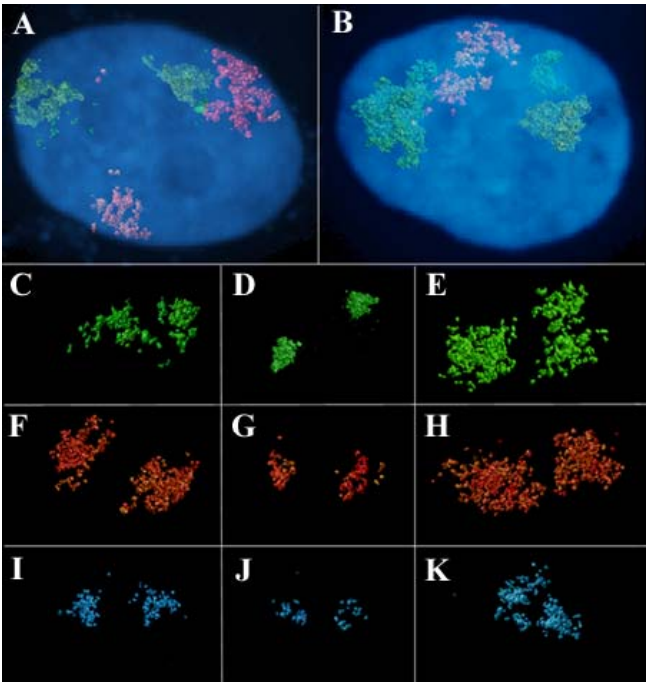

Fig. 3. Images of CTs after deconvolution and segmentation. CTs of chromosomes 1 (green), 2 and 13 (red) touch the nuclear periphery (A, B). The row of green images (C, D, and E) demonstrates representative structural changes of CTs of chromosome 1 after lamin A/C silencing (D) and cytochalasin D treatment (E) compared with a control cell (C). Similarly, the row of red images (F, G, and H) shows changes of chromosome 2 CTs and the row of blue images (I, J, and K) shows changes of chromosome 13 CTs.

Changes in differentiation of granulocytes in acute myeloid leukemia

Our earlier results show that human terminally differentiated neutrophils of peripheral blood do not have HP1 proteins even if their nuclear chromatin is highly condensed. The high chromatin condensation of these cells is accompanied by extremely high level of dimethylated histone H3 on lysine 9 (H3K9), however contrary to other terminally differentiated cells this methylated lysine is not accessible for immunodetection. In chronic phase of chronicle myeloid leukemia (CML), the characteristics of blood neutrophils are similar as that of healthy neutrophils; there is the absence of HP1 proteins and contrary to healthy neutrophils, a very low level of

immunodetected dimethylated H3K9 could be detected, indicating some small changes in chromatin condensation. However, as the disease accelerates and proceeds to the blast crises, the immunodetected level of dimethylated H3K9 became high together with appearance of the HP1 proteins, especially HP1 β and γ. The monitoring of the process of blood stem cells myeloid differentiation ex vivo in the presence of cytokines and Granulocyte Colony-Stimulating Factor (G-CSF) showed that HP1 proteins are eliminated from heterochromatin of stem cells soon after the cessation of their proliferation. This changeover is accompanied also by decrease of H3K9 accessibility for immunodetection and in the end of this process neither HP1 nor dimethylated H3K9 are detected in final differentiated neutrophils.

In collaboration with the Hemato-oncological Clinic of the Faculty Hospital in Olomouc we started to study the status of HP1 proteins and dimethylated H3K9 also in neutrophils of acute myeloid leukemia (AML) with normal karyotype. It is a recently find form of AML provoked by mutations in the gene for nucleophosmin (NPM1). The favorable prognosis of this disease is dependent on mutations in additional genes. The most frequent collateral mutations arise in the FLT3 (fms-like-tyrosinkinase) gene and they aggravate the prognosis. Our results show that neutrophils of all patients with some mutation in NPM1 gene, analyzed until now, have small amount of HP1β and immunodetected dimethylated H3K9 (exceptionally HP1γ but never HP1α) in their chromatin regardless mutations in additional genes. It follows from this finding that terminal differentiation of neutrophils of these patients is impaired resulting in incomplete condensation of their chromatin. We suppose that in the case of the successful treatment of this disease, the terminal differentiation of neutrophils will be complete which will be manifested by disappearance of HP1 proteins from their chromatin.

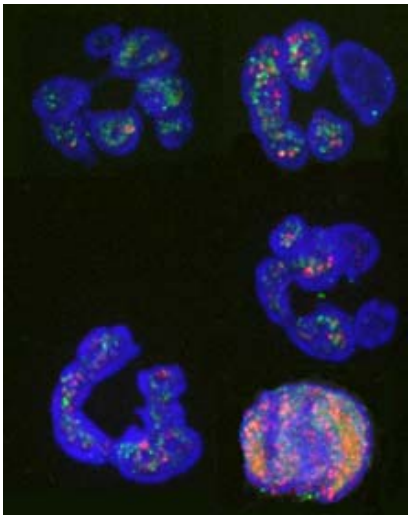

Fig. 4. Neutrophils of an AML patient having mutations in NPM1 and FLT3 genes. Immunodetection shows the presence of HP1β (red) and HP1γ (green) proteins. Much higher level of these proteins could be seen in a lymphocyte on the bottom of the image.

Chromatin structure in two regions of chromosome 11 differing fundamentally in the content of highly expressed genes

The transcriptom map of Caron et al. (Science 291,2001,1289-1292) shows significant clustering of highly expressed genes with patterns that are remarkably similar among many cell or tissue types. The most highly expressed genes tend to be clustered in genomic regions called RIDGEs (regions of increased gene expression). ANTI-RIDGEs regions have low gene density and contain mainly weakly expressed genes.

In this work we tried to find the nuclear structure and arrangement of chromatin in one RIDGE and one ANTI-RIDGE of chromosome 11 by the measuments of distances between probes in both regions visualized by 3D FISH in G1 nuclei of human fibroblasts. To get the most precise information about the structure of chromatin in these regions, the statistic analysis of large number of measurements was performed owing to the variable character of chromatin in individual cells.

The measurements of nuclear distances between the high number of DNA

probes in these regions show that chromatin in both regions exhibits the random walk behavior typical of linear flexible molecular chain folded in a random manner. The relationship between mean-square interphase distances and genomic distances between probes has two linear phases with transition at ~ 2 Mbp showing the existence of two levels of chromatin organization in both regions. One, at lower genomic distances is looser and that at distances up to ~ 2 Mbp is more tight. However the chromatin compaction in both levels of its organization is of about 40 % lower in the region rich on highly expressed genes – RIDGE. Thus, the results show that the three dimensional structure of interphase chromosome underlie to the same rules regardless the local distribution and content of highly expressed genes. However the distribution and content of highly expressed genes determine the degree of chromatin compaction in the chromosome region being more open in the region of clusters of highly expressed genes.

Granted projects

GA CR 204/06/0978, Post-translation modification of histones after cell treatment by inhibitors of histon deacetylases and during cell differentiation. Principal investigator: E. Bártová, 2006 - 2008

LC06027, Center of basic research for monoclonal gamapathy of multiple myeloma. Principal investigator: R. Hájek, co-investigator: E. Bártová, 2006 - 2010

AS CR 1QS500040508, Methylation of histone H3 as a prognostic marker of chronic myeloid leukemia remission. Principal investigator: S. Kozubek, 2005 – 2009

GA CR 204/06/P349, Dynamic structure and function of the cell nucleus associated with DNA breaks. Principal investigator: M. Falk, 2006-2008

6. FP EU, LSHG-CT-2003-503441, 3D Genome structure and function. Principal investigator: R. van Driel, Co-principal investigator: S. Kozubek, 2004-2007

GA AS CR A5004306, Structure of human genome. Principal investigator: S. Kozubek, 2004-2008

ME, COST 1P050C084, Dynamic structure and function of the cell nucleus after irradiation, principal investigator: S. Kozubek, 2005-2007

ME, LC 535, Center of Basic Research, Dynamics and organization of chromosomes during the cell cycle. principal investigator: I. Raška, Co-principal investigator: S. Kozubek, 2005-2009

Publications

Bártová, E., Pacherník, J., Kozubík, A., Kozubek, S.: *Differentiation-specific association of HP1alpha and HP1beta with chromocentres is correlated with clustering of TIF1beta at these sites.* Histochem Cell Biol. 127, 2007, 375-388.

Fojtová, M., Piskala, A., Votruba, I., Otmar, M., Bártová, E., Kovařík, A.: *Efficacy of DNA hypomethylating capacities of 5-aza-2'-deoxycytidine and its alpha anomer.* Pharmacol. Res. 55, 2007, 16-22.

Koutná, I., Krontorád, P., Svoboda, Z., Bártová, E., Kozubek, M., Kozubek, S.: *New insights into gene positional clustering and its properties supported by large-scale analysis of various differentiation pathways.* Genomics 89, 2007, 81-88.

Kroupová, J., Bártová, E., Fojt, L., Strašák, L., Kozubek, S., Vetterl, V.: *Low-frequency magnetic field effect on cytoskeleton and chromatin.* Bioelectrochemistry 70, 2007, 96-100.

Bártová, E., Krejčí, J., Harničarová, A., Kozubek, S.: *Differentiation of human embryonic stem cells induces condensation of chromosome territories and formation of heterochromatin protein 1 foci*. Differentiation. 76, 2008, 24-32.

Bártová, E., Harničarová, A., Krejčí, J., Strašák, L., Kozubek, S.: *Single-cell c-myc gene expression in relationship to nuclear domains.* Accepted for publication in Chromosome Research, 16, 2008, 325-343.

Galiová, G., Bártová, E., Raška, I., Krejčí, J., Kozubek, S.: *Chromatin changes induced by lamin A/C deficiency and the HDAC inhibitor TSA.* Accepted for publication in European Journal of Cell Biology, 2008.

Falk, M., Lukášová, E., Gabrielová, B., Ondřej, V., Kozubek, S.: *Chromatin dynamics during DSB repair.* Biochim Biophys Acta 1773, 2007, 1534-1545.

Ondřej, V., Lukášová, E., Falk, M., Kozubek, S.: *The role of actin and microtubule networks in plasmid DNA intracellular trafficking*. Acta

Biochim. Pol. 54, 2007, 657-663.

Vařecha, M., Amrichová, J., Zimmermann M., Ulman, V., Lukášová, E., Kozubek, M.: *Bioinformatic and image analyses of the cellular localization of the apoptic proteins endonuclease G, AIF, and AMID during apoptosis in human cells*. Apoptosis 12, 2007, 1155-1171.

Vávrová, J., Janovská, S., Řezáčová, M., Hernychová, L., Tichá, Z., Vokurková D., Záškodová, D., Lukášová, E.: *Proteomic analysis of MOLT-4 cells treated by valproic acid.* Mol. Cell Biochem 303, 2007, 53-61.

Goetze, S., Mateos-Langerak, J., Gierman, H., de Leeuw, W., Giromus, O., Indemans, M.H.G., Koster, J., Ondřej, V., Verstieg, R., van Driel, R.: *The three-dimensional structure of human interphase chromosomes is related to the transcriptom map.* Mol. Cell Biol. 27, 2007, 4475-4487.